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13. ABSTRACT (Maximum 200 Words)
Epidemiological studies suggest that dietary folate intake and blood levels of folate are inversely related to breast cancer risk. Because only few modifiable risk factors for breast cancer exist, the role of folate in modifying breast cancer risk merits further consideration. Folate is an ideal agent for chemoprevention of breast cancer. It is a natural vitamin, inexpensive, virtually free of side effects, and possesses biologically plausible mechanisms for cancer prevention. However, folate appears to possess dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention. Folate deficiency has an inhibitory, whereas folate supplementation has a promoting, effect on progression of established neoplasms. By contrast, folate deficiency in normal tissues predisposes them to neoplastic transformation, and modest levels of folate supplementation suppress, whereas supraphygiologic doses enhance, the development of tumors in normal tissues. Therefore, the potential effect of folate chemoprevention needs to be clearly established in appropriate animal models before folate supplementation can be considered in humans. Given these considerations, this proposal investigates the effects of dietary folate deficiency and supplementation on mammary tumorigenesis and potential molecular and cellular mechanisms by which folate modulates mammary tumorigenesis in the well established carcinogen rat model of breast cancer.

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INTRODUCTION

Breast cancer is the most common cancer and the second leading cause of cancer deaths in women in the United States (*1*). Genetic predisposition (*2-4*) and hormonal/reproductive factors (*5-7*), two important determinants of breast cancer risk, are not readily modifiable. Therefore, much effort has been directed towards identifying potentially modifiable dietary and lifestyle factors that would lead to the prevention of breast cancer. Although epidemiological and animal studies have suggested that dietary factors, such as fat, fiber, vegetables and fruits, antioxidants and alcohol, may influence breast cancer risk, effects of these factors on breast cancer risk are inconsistent and contradictory (*1*). As such, prevention of breast cancer through dietary modifications remains an elusive and challenging task.

Folate, a water-soluble B-vitamin and important co-factor in one-carbon metabolism, has recently been identified as an important nutritional factor that may modulate carcinogenesis (*8-10*). The role of folate in carcinogenesis has been best studied for colorectal cancer (*8-10*). The majority of over 25 published epidemiological studies indicate that dietary folate intake and blood folate levels are inversely associated with colorectal cancer risk (*8-10*). Collectively, these studies suggest an ~40% reduction in the risk of colorectal neoplasms in subjects with highest dietary folate intake compared with those with the lowest intake (*8-10*). These studies also suggest that a modest reduction in folate status is sufficient to enhance colorectal cancer risk (*8-10*). Animal studies have also been generally supportive of a causal relationship between folate depletion and colorectal cancer risk as well as a dose-dependent protective effect of modest levels of dietary folate supplementation (4-10X) above the basal dietary requirement on the development and progression of colorectal neoplasms (*11-15*). Animal studies have also shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels (*12, 16, 17*) and folate intervention after microscopic neoplastic foci are established in the colorectal mucosa (*13, 14*) promote, rather than suppress, colorectal carcinogenesis. An accumulating body of evidence suggests that folate status may also play a modulatory role in the development of several other malignancies (e.g. lung, pancreas, stomach, cervix, esophagus, brain and leukemia) (*8-10*). The precise nature and magnitude of the relationship between folate status and the risk of these malignancies, however, are less clearly defined compared with colorectal cancer.

The relationship between folate status and breast cancer risk has just begun to be reported in the epidemiological literature. Among dietary factors implicated in the development of breast cancer, the inverse relationship between the consumption of vegetables and fruits (the major source of dietary folate) and breast cancer risk (*18*) and the positive correlation between the intake of alcohol (folate antagonist) and breast cancer risk (*19, 20*) have been most consistent (*1*). Among 9 published case-control studies that investigated the relationship between dietary folate intake and breast cancer risk, 7 showed either a significant or equivocal inverse relationship that was not statistically significant, that became nonsignificant after adjustment, or that could not be distinguished from other factors in their relation to risk (*21-27*), whereas 2 showed an unequivocal null association (*28, 29*). In some studies, the observed inverse association was further modified by the intake of alcohol and other folate cofactors (e.g. methionine, vitamins B_6 and B_{12}) (*24, 26, 27*). One nested case-control study, using stored serum samples, found no association between serum folate and breast cancer risk (*30*). Two large prospective studies have shown a weak inverse association between the total or dietary intake of folate and breast cancer risk (*31, 32*). These prospective studies, however, have indicated that

low intakes of folate increase, whereas high intakes of folate decrease, breast cancer risk among women who regularly consume alcohol (*31, 32*), supporting folate-alcohol interactions in breast carcinogenesis observed in case-control studies (*24, 26, 27*).

Two animal studies published to date have produced conflicting results concerning the effect of folate on mammary tumorigenesis. In mice with confirmed spontaneous mammary cancers, daily intravenous injections of fermentation *Lactobacillus casei* factor (pteroyltriglutamate) significantly regressed mammary tumors and decreased new mammary tumor formation and lung metastases (*33*). Another study employing the *N*-methyl-*N*-nitrosourea (MNU) rat model showed that a folate-deficient diet provided during the initiation phase of mammary tumorigenesis significantly reduced tumor multiplicity and increased tumor latency compared with a control and folate-supplemented diet (*34*). The incidence of mammary tumors, however, was not significantly different among these groups (*34*). Several inherent limitations associated with these animal studies, however, preclude a definitive conclusion concerning the effect of folate on mammary tumorigenesis.

Because only few modifiable risk factors for breast cancer exist, recent epidemiological observations which suggest that folate deficiency increases, whereas supplementation reduces, breast cancer risk merit further consideration. Folate is an ideal agent for potential chemoprevention of breast cancer. It is a natural vitamin, inexpensive, virtually free of side effects (*35*), and possesses biologically plausible mechanisms for cancer prevention (*8-10*). However, the results from published epidemiological and animal studies have been neither consistent nor convincing. Furthermore, a growing body of evidence suggests that folate possesses the dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention (*11-17*). Folate deficiency has an inhibitory, whereas folate supplementation has a promoting, effect on progression of established neoplasms (*11-17*). By contrast, folate deficiency in normal epithelial tissues appears to predispose them to neoplastic transformation, and modest levels of folate supplementation suppress the development of tumors in normal tissues (*11-17*). Therefore, the potential effect of folate chemoprevention needs to be clearly elucidated in appropriate animal models before folate supplementation can be considered in humans. Given these considerations, we proposed to study the effects of dietary folate deficiency and supplementation on the development and progression of mammary tumors in the well-established MNU rat model of breast cancer in three animal experiments and to investigate potential molecular mechanisms by which dietary folate modulates mammary tumorigenesis. Notwithstanding the limitations associated with animal models, the MNU rat model is widely used to determine the effects of dietary factors on mammary tumorigenesis for the following reasons: (a) histological similarities of adenocarcinomas to human breast cancer; (b) local invasiveness and metastatic potential; (c) a clear operational distinction between the initiation and promotion stages; and (d) hormonally dependent mammary tumorigenesis (*36-40*).

BODY

Task 1 (Specific Aim I): To determine whether sustained folate deficiency of a moderate degree enhances, and whether a modest degree of folate supplementation above the basal requirement suppresses, the development of mammary tumors in the MNU rat model of mammary carcinogenesis (*initiation + promotion combined*)

a. Animal experiment (feeding + MNU injection + sacrifice + sample harvesting) [months 1 – 8]
b. Preparation of samples (paraffin embedding, preparation of slides and staining of tumors and adjacent normal tissues, DNA and RNA extraction, tissue folate extraction) [months 8 – 9]
c. Histologic analysis of tumors, microdissection of neoplastic foci and adjacent normal tissues for DNA extraction, folate analysis [months 9 – 11]
d. Data analysis [months 11 – 12]

All specific tasks outlined in Task 1 were essentially completed during the first year of funding (August 1, 2001 – July 31, 2002). We investigated the effect of dietary folate deficiency and supplementation on the development and progression of mammary tumors in the MNU rat model. Weaning, female Sprague-Dawley rats were fed diets containing either 0 mg (deficient; n=22), 2 mg (basal dietary requirement, control; n=20) or 8 mg (supplemented; n=20) folate /kg diet for 30 weeks. At 50 days of age, rats received an intraperitoneal injection of MNU (50 mg/kg body weight). Body weights were recorded weekly. The daily food consumption of each group was measured on a predetermined day of each week. All rats were palpated for mammary tumors once a week beginning 4 weeks after MNU administration. The number, size and location of each tumor were recorded in a manner that, after histological diagnosis, the time of appearance of the cancers could be determined. All the rats were monitored daily for clinical evidence of illness or morbidity and those approaching a moribund state were promptly euthanized. In addition, rats with tumor burden exceeding 10% of body weight, tumors >15-20 mm in diameter, tumors that impaired normal movement of the animals, and ulcerating tumors were immediately euthanized during the study. Blood was collected from the tail of each rat within a week of MNU injection and from the heart at necropsy for the serum folate assay. Given the latency period of 3-6 months associated with a single intraperitoneal MNU injection and the average duration for the systemic and tissue folate levels to stabilize, the rats were sacrificed by carbon dioxide inhalation followed by cervical dislocation at 23 weeks after MNU injection (27 weeks after dietary intervention or 30 weeks of age). The liver from each rat was harvested for hepatic folate concentration determination. All macroscopic mammary tumors were counted, excised and weighed, and diameters of each tumor were measured using a digital caliper for final tumor volume computation in a blinded fashion. One-half of each macroscopic tumor was processed for DNA extraction. The other half of the tumor was processed in a standard manner for histological analysis according to Russo et al. (*39*) by three experienced pathologists blinded to the study group independently. In the case of a discrepancy, two similar interpretations were utilized for the final analysis. Normal mammary tissue was processed for DNA extraction and mammary tissue folate determination. Between-group comparisons of continuous variables were assessed using the Kruskal-Wallis and Mann-Whitney non-parametric tests. For categorical

response variables, differences among the groups were assessed by Pearson chi-square test. The Kaplan-Meier survival analysis and the Log Rank test were used to compare the rates of tumor appearance among the three groups. All significance tests were two sided and were considered statistically significant if the observed significance level was <0.05. Results are expressed as mean ± SEM. Statistical analyses were performed using SPSS (version 10).

Serum folate concentrations accurately reflected dietary folate levels at the time of MNU administration and at necropsy (P<0.001). The mean folate concentrations of the normal mammary glad of the folate-deficient group were significantly lower than those of the control and folate-supplemented groups (P<0.001), whereas no significant differences between the control and folate-supplemented groups was observed. The final incidence of mammary tumors in the folate-deficient group was significantly lower than that of the control and folate-supplemented groups (55% versus 90% and 75%, respectively, P=0.04). Kaplan-Meier analyses also demonstrated similar cumulative tumor incidence trends (P=0.06). By contrast, dietary folate supplementation did not significantly modulate both the final and cumulative incidences of mammary tumors compared with the control group. Dietary folate status had no significant effect on mean volume, weight, latency or multiplicity of mammary tumors. These data suggest that dietary folate deficiency of a moderate degree suppresses mammary tumorigenesis in this model. In contrast, dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis. These observations contradict the generally accepted notion based on epidemiologic evidence, which suggests that folate deficiency enhances, whereas folate supplementation suppresses, the development of breast cancer. Notwithstanding the limitations associated with this model, our data suggest that the role of folate in mammary tumorigenesis needs to be clarified in future studies for safe and effective prevention of breast cancer.

During the second year of funding (August 1, 2002 – July 31, 2003), one abstract arising from Task 1 entitled "Dietary folate deficiency suppresses mammary tumorigenesis in a chemical carcinogen rat model of breast cancer" was presented in Poster Session: Chemoprevention, P13-8 at the Era of Hope meeting in Orlando, Florida on September 26, 2002 (**Appendix 1**). One manuscript arising from Task 1 entitled "Dietary folate deficiency suppresses N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats" was published in Carcinogenesis (*41*) (**Appendix 2**).

Task 2 (Specific Aim II): To determine whether folate deficiency enhances, and whether folate supplementation suppresses, the *initiation* of mammary carcinogenesis

a. Animal experiment (feeding + MNU injection + sacrifice + sample harvesting) [months 4 – 11]
b. Preparation of samples (paraffin embedding, preparation of slides and staining of tumors and adjacent normal tissues, DNA and RNA extraction, tissue folate extraction) [months 11 – 12]
c. Histologic analysis of tumors, microdissection of neoplastic foci and adjacent normal tissues for DNA extraction, folate analysis [months 12 – 14]
d. Data analysis [months 14 – 15]

During the first year of funding, we began this animal experiment and all specific tasks outlined in Task 2 were completed during the second year of funding. In this animal experiment, we investigated the effect of dietary folate deficiency and supplementation on the *initiation* phase of mammary tumorigenesis in the MNU rat model. Based on the data obtained from Task 1, it is important to determine whether the suppressive effective of dietary folate deficiency on mammary tumorigenesis in this model is during the initiation and/or promotion/progression phases of mammary tumorigenesis. In this experiment, similar to the animal experiment in Task 1, weaning, female Sprague-Dawley rats were fed diets containing either 0 mg (deficient; n=21), 2 mg (basal dietary requirement, control; n=20) or 8 mg (supplemented; n=20) folate /kg diet. At 50 days of age, rats received an intraperitoneal injection of MNU (50 mg/kg body weight). The initial diets were terminated one week following MNU-injection and all the rats were placed on the control (2 mg folate/kg diet) diet until the time of sacrifice (30 weeks of age). Animals were maintained and sacrificed, samples were harvested and prepared, and all assays and analyses were performed in the same manner as described in Task 1 (*41*).

Serum folate concentrations accurately reflected dietary folate levels at the time of MNU administration ($P<0.001$). At necropsy, serum and mammary gland folate concentrations of the three groups were not significantly different because the animals were placed on on the control diet for 23 weeks after the MNU administration at 50 days of age. Dietary folate status during the initiation phase of MNU-induced mammary tumorigenesis had no significant effect on final and cumulative incidences, latency, volume, weight or multiplicity of mammary tumors. These data suggest that dietary folate status does not modulate the initiation phase of MNU-induced mammary tumorigenesis. One explanation for this observation is that, although the dose and route of MNU administration employed in this study may be appropriate in studies examining the effect of other potential chemopreventive agents in this model, the effect may be too overwhelmingly carcinogenic for folate to modulate. Therefore, the effect observed with dietary folate in Task 1 may be predominantly on promotion and progression, not on initiation, of MNU-induced neoplastic foci.

The data from Task 2 will be combined with the data from Task 3 and a manuscript will be prepared as discussed in the next section.

Task 3 (Specific Aim III): To determine whether folate deficiency enhances, and whether folate supplementation suppresses, the *promotion* of mammary carcinogenesis

e. Animal experiment (feeding + MNU injection + sacrifice + sample harvesting) [months 7 – 14]
f. Preparation of samples (paraffin embedding, preparation of slides and staining of tumors and adjacetn normal tissues, DNA and RNA extraction, tissue folate extraction) [months 14 – 15]
g. Histologic analysis of tumors, microdissection of neoplastic foci and adjacent normal tissues for DNA extraction, folate analysis [months 15 – 17]
h. Data analysis [months 17 – 18]

During the first year of funding, we began this animal experiment and all specific tasks outlined in Task 3 were completed during the second year of funding. In this animal experiment, we investigated the effect of dietary folate deficiency and supplementation on the *promotion/progression* phase of mammary tumorigenesis in the MNU rat model. Based on the data obtained from Task 1, it is important to determine whether the suppressive effective of dietary folate deficiency on mammary tumorigenesis in this model is during the initiation and/or promotion/progression phases of mammary tumorigenesis. In this experiment, similar to the animal experiments in Task 1 and 2, weaning, female Sprague-Dawley rats (n=93) were placed on the control diet (2 mg folate/kg diet). At 50 days of age, rats received an intraperitoneal injection of MNU (50 mg/kg body weight). One week following MNU administration, rats were randomized to receive diets containing either 0 mg (deficient; n=33), 2 mg (basal dietary requirement, control; n=30) or 8 mg (supplemented; n=30) folate /kg diet until the time of sacrifice (30 weeks of age). Animals were maintained and sacrificed, samples were harvested and prepared, and all assays and analyses were performed in the same manner as described in Task 1 and 2 (*41*).

Serum folate concentrations of the three groups were not significantly different at the time of MNU-administration after being on the control diet for 50 days. However, at necropsy (30 weeks of age), serum folate concentrations of the three groups accurately reflected dietary folate levels after being placed on three diets containing different amounts of folate for 23 weeks after MNU-administration ($P<0.001$). The mean folate concentrations of the normal mammary glad of the folate-deficient group were significantly lower than those of the control and folate-supplemented groups ($P<0.001$), whereas no significant differences between the control and folate-supplemented groups was observed. The folate-deficient diet provided during the promotion phase of MNU-induced mammary tumorigenesis significantly reduced the final incidence (48% versus 80% and 78%, respectively, $P<0.03$), cumulative incidence ($P<0.03$) and volume ($P<0.01$) of adenocarcinomas compared with the control and folate-supplemented diets, whereas no significant effects were observed for latency, weight or multiplicity. Folate supplementation provided during the promotion phase had no significant effect on incidence and any of the parameters of adenocarcinomas compared with the control diet. Dietary folate status during the promotion phase had no significant effect on incidence and any of the parameters of adenomas. These data collectively suggest that dietary folate deficiency of a moderate degree significantly suppresses the promotion/progression of MNU-induced mammary neoplastic foci to adenocarcinomas, whereas dietary folate supplementation does not significantly modulate mammary tumorigenesis in this model.

Taken together, the data from Task 1, 2 and 3 collectively suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats and this effect is predominantly on the promotion/progression phase of MNU-induced mammary tumorgenesis. These observations suggest that the conventional dose and route of MNU injection employed in these studies have created an overwhelmingly carcinogenic milieu for folate status to modulate initiation of mammary tumorigenesis. Regardless of the levels of dietary folate, MNU induced and established neoplastic foci in mammary tissues. In this setting, folate deficiency suppressed the progression of and/or caused regression of established mammary neoplastic foci. This explanation is consistent with the prior observations made in Min and *Apc+/-Msh2-/-* mice with respect to intestinal tumorigenesis (*13, 14*). Therefore, the inhibitory

effect of folate deficiency on MNU-induced mammary tumorigenesis in this rat model is primarily on promotion/progression of established mammary neoplastic foci. These animal studies in conjunction with prior observations made in animal models of colorectal cancer (*13, 14*) suggest that folate deficiency has an inhibitory effect on progression of established neoplasms. In contrast, folate supplementation may promote the progression of established intestinal neoplastic foci (*13, 14*), whereas the same degree of folate supplementation did not promote the progression of established MNU-induced mammary neoplastic foci in our studies. Future studies are warranted to clarify the role of folate status in prevention and treatment of breast cancer.

A manuscript incorporating the data from Task 2 and 3 will be prepared and submitted to Cancer Research by the end of September 2003. The tentative title of this manuscript is "Effects of dietary folate on the initiation and promotion phases of MNU-induced mammary tumorigenesis in rats" and the authors are "Kotsopoulos, J, Sohn K-J, Martin R, Renlund R, Hwang S, Medline A, and Kim YI."

Task 4 (Specific Aim IV): To determine molecular mechanisms by which folate status modulates mammary tumorigenesis in this model (using samples predominantly from Task 1)

a. Genomic DNA methylation and site-specific CpG-rich promoter methylation (ER, p16, p53 genes) [months 10 – 14]
b. Development of immunochemical staining with anti-methylcytosine antibody [months 10 –12]
c. Immunohistochemical determination of expression of ER, p16, p53 proteins [months 12 –14]
d. RNase proteciton assay to determine steady-state mRNA levels of DNA methyltransferases (Dnmt1, Dnmt3a, Dnmt3b) and demethylase [months 14 – 16]
e. Determination of DNA methyltransferase and demethylase activities [months 16 – 18]
f. Immunohistochemical determination of expression of Dnmt1, Dnmt3a, Dnmt3b and demethylase [months 18 – 20]
g. Genomic and site-specifc p53 DNA strand breaks [months 20 – 22]
h. Microsatellite instability [months 22 – 25]
i. PCR-RFLP/liquid hybridization to detect Ha-ras mutations [months 25 –27]
j. Immunostaining/PCR-SSCP/direct sequencing to detect p53 mutations [months 27 – 29]
k. PCNA and apoptosis [months 29 – 31]
l. Immunochemical detection of cyclin D1 overexpression [months 31 – 32]
m. Data analysis [months 32 – 34]

During the first year of funding, we have completed task (a) of Task 4. DNA from normal mammary tissues and mammary tumors was extracted by a standard technique using a lysis buffer containing proteinase K followed by phenol, chloroform, and isoamyl alcohol organic extraction (*42*). The size of DNA estimated by agarose-gel electrophoresis was >20 kb in all instances. No RNA contamination was detected on agarose-gel electrophoresis. The final

preparations had a ratio of A_{260} to A_{280} between 1.8 and 2.0. The concentration of each DNA sample was determined as the mean of 3 independent spectrophotometric readings. The methylation status of cytosine-guanine (CpG) sites in genomic DNA from normal mammary tissues and mammary tumors was determined by the in vitro methyl acceptance capacity of DNA using [^{3}H-methyl]S-adenosylmethionine (SAM) as a methyl donor and a prokaryotic CpG DNA methyltransferase, Sss1, as previously described (*12, 14, 43, 44*). The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous [^{3}H-methyl] incorporation. Briefly, mammary tumor and non-neoplastic mammary gland DNA (500 ng) was incubated with 2.0 μCi of [^{3}H-methyl]SAM (New England Nuclear, Boston, MA), 3 units of Sss1 methylase (New England Biolabs, Beverly, MA), and 1X Sss1 methylation buffer [120mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM EDTA, 1 mM dithiothreitol] in a total volume of 30 μL for 1 hour at 30°C. Sss1 was inactivated by incubating at 65°C for 10 minutes. The in vitro methylated DNA was isolated from a 15 μl aliquot of the reaction mixture by filtration on a Whatman DE-81 ion-exchange filter (Fisher Scientific, Springfield, NJ). The DNA was washed three times with 0.5 M sodium phosphate buffer (pH 7.0), air-dried and the radioactivity of the DNA retained in the filters was measured by scintillation counting using a nonaqueous scintillation fluor. The amount of radiolabel bound to a filter from an incubation mixture without DNA (control) was used as background and was subtracted from the values obtained with mixtures containing DNA. The background value was always <1% of the uptake observed with DNA samples. All analyses were performed in duplicate. Differences in genomic DNA methylation between normal mammary gland and tumor in each diet group was assessed by the Wilcoxon signed ranks test.

The degree of ^{3}H-methyl incorporation into DNA of the mammary adenocarcinoma and into DNA from the pair-matched non-neoplastic mammary tissue was not significantly different among the three dietary groups. However, the degree of ^{3}H-methyl incorporation into DNA of the mammary adenocarcinomas, which is inversely related to the extent of genomic DNA methylation, was 4- to 5-fold higher than that of non-neoplastic mammary tissue within each dietary group, indicating a significantly lower degree of genomic DNA methylation in the adenocarcinomas compared with the normal mammary tissue ($P<0.03$). DNA methylation is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in chromatin modifications and in the development of mutations (*45, 46*). Neoplastic cells simultaneously harbor widespread genomic DNA hypomethylation and more specific regional areas of hypermethylation (*45, 46*). Genomic and protooncogene-specific hypomethylation appears to be an early, and consistent, event in carcinogenesis (*45, 46*). There appears to be a direct correlation between the extent of genomic DNA hypomethylation and tumor progression (*45, 46*). Genomic hypomethylation results in genomic instability and increased mutations, and protooncogene-specific hypomethylation results in increased gene expression (*45, 46*). In addition, site-specific hypomethylation at the promoter region of tumor suppressor and mismatch repair genes is an important mechanism in gene silencing in carcinogenesis (*45, 46*). Although promoter CpG islands hypermethylation of several genes including *BRCA1, ER, p16, E-Cadherin, TMS1, RASSF1*, leading to inactivation of these genes have been observed in human breast cancer (*47-52*), very few studies have reported genomic hypomethylation in human breast cancer (*53, 54*). To our knowledge, our study is the first to demonstrate that genomic DNA hypomethylation is an epigenetic phenomenon associated with MNU-induced mammary tumorigenesis in rats. The extent of genomic DNA methylation in

mammary adenocarcinomas and in nonneoplastic mammary tissues was not significantly modulated by folate status. This observation suggests that altered genomic DNA methylation was not a likely mechanism by which folate deficiency suppressed mammary tumorigenesis in our study. Folate, in the form of 5-methyltetrahydrofolate, is involved in remethylation of homocysteine to methionine, which is a precursor of SAM, the primary methyl group donor for most biological methylation reactions (*8-10*). Although isolated folate deficiency has been shown to induce genomic DNA hypomethylation in circulating lymphocytes in humans (*55, 56*), this effect has not been demonstrated in the colorectum or liver in rodents fed the same folate-deficient diet employed in the present study (*12, 14, 57*). However, an extremely severe degree of folate deficiency has been shown to induce genomic (*43*) and site-specific *p53* (*44*) DNA hypomethylation in rat liver, although other studies have disputed the finding on genomic DNA methylation (*44*). Because both site-specific hypo- and hypermethylation play a role in carcinogenesis (*45, 46*) and because folate may modulate DNA methylation in a site-specific manner (*44*), it would be of great interest to study site-specific methylation of protooncogenes and tumor suppressor genes implicated in mammary tumorigenesis (*2-4*) and the effect of folate status.

The data concerning genomic mammary DNA methylation in MNU-mammary tumorigenesis in rats were incorporated into the published manuscript from Task 1 (*41*) (**Appendix 2**). During the second year of funding, we also examined the effect of dietary folate on genomic DNA methylation in mammary gland tissues from Task 2 and Task 3. Similar to the observations made in Task 1, the degree of ^{3}H-methyl incorporation into DNA of the mammary adenocarcinoma and into DNA from the pair-matched non-neoplastic mammary tissue was not significantly different among the three dietary groups. However, the degree of ^{3}H-methyl incorporation into DNA of the mammary adenocarcinomas, which is inversely related to the extent of genomic DNA methylation, was 4- to 5-fold higher than that of non-neoplastic mammary tissue within each dietary group, indicating a significantly lower degree of genomic DNA methylation in the adenocarcinomas compared with the normal mammary tissue ($P<0.03$). Given the fact that the extent of genomic DNA methylation in mammary adenocarcinomas and in nonneoplastic mammary tissues was not significantly modulated by folate status, altered genomic DNA methylation was not a likely mechanism by which folate deficiency suppressed mammary tumorigenesis in these experiments. These observations will be incorporated into the aforementioned manuscript being prepared for Task 2 and 3 at present. At present, we are working on site-specific DNA methylation of the ER, p16 and p53 genes and depending on the results of this analysis and we will proceed to determine protein expression of ER, p16 and p53. Furthermore, we are working on RNA and protein expression and activity of Dnmt1, Dnmt3a, Dnmt3b and demethylase (specific tasks d – f) at present. The remaining specific tasks in Task 4 will be performed during the third year of funding.

KEY RESEARCH ACCOMPLISHMENTS

1. Our data from Task 1, 2 and 3 collectively indicate that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats and that this effect is predominantly on the promotion/progression phase of MNU-induced mammary tumorigenesis. Our data also indicate that dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis in this model. These observations contradict the generally accepted notion based on epidemiologic evidence, which suggests that folate deficiency enhances, whereas folate supplementation suppresses, the development of breast cancer in humans. Notwithstanding the limitations associated with this model, our data suggest that the role of folate in mammary tumorigenesis needs to be clarified in subsequent animal and human studies for safe and effective prevention of breast cancer.
2. Our study is the first to demonstrate that genomic DNA hypomethylation is an epigenetic phenomenon associated with MNU-induced mammary tumorigenesis. However, the extent of genomic DNA methylation in mammary tumors was not significantly modulated by folate status. This observation suggests that altered genomic DNA methylation was not a likely mechanism by which folate deficiency suppressed mammary tumorigenesis in this study.

REPORTABLE OUTCOMES

1. Kotsopoulos J, Sohn K-J, Martin R, Renlund R, McKerlie C, Hwang S, Medline A, **Kim YI**. Dietary folate deficiency suppresses mammary tumorigenesis in a chemical carcinogen rat model of breast cancer. (poster presentation at Chemoprevention, P13-8 at Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orlando, Florida, September 25 – 28, 2002; **Appendix 1**)
2. Kotsopoulos J, Sohn K-J, Martin R, Choi M, Renlund R, McKerlie C, Hwang S, Medline A, **Kim YI**. Dietary folate deficiency suppresses N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats. Carcinogenesis 2003; 24: 937 – 944 (**Appendix 2**)

CONCLUSIONS

Our data from Task 1, 2 and 3 collectively indicate that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats and that this effect is predominantly on the promotion/progression phase of MNU-induced mammary tumorigenesis. Our data also indicate that dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis in this model. These observations contradict the generally accepted notion based on epidemiologic evidence, which suggests that folate deficiency enhances, whereas folate supplementation suppresses, the development of breast cancer in humans. Notwithstanding the limitations associated with this model, our data suggest that the role of folate in mammary tumorigenesis needs to be clarified in subsequent

animal and human studies for safe and effective prevention of breast cancer. Future studies employing lower doses of MNU, lower fat content and higher levels of folate supplementation may be necessary to clearly elucidate the effect of folate on mammary tumorigenesis in this model. The effect of folate on mammary tumorigenesis observed in the present study needs to be confirmed in other animal models. Given the possible interactions of folate with alcohol and other folate co-factors in modifying breast cancer risk observed in some epidemiological studies, these potential interactions merit further investigation. Our data concerning genomic DNA methylation also indicate that genomic mammary DNA hypomethylation is an epigenetic mechanism by which mammary tumors develop in the MNU rat model. Studies are underway to investigate site and gene-specific DNA methylation changes in this model. Our data also suggest that changes in genomic mammary DNA methylation are not a likely mechanism by which folate modulates mammary tumorigenesis in this model. Studies are underway to investigate other molecular mechanisms by which dietary folate deficiency suppresses mammary tumorigenesis in this model as outlined in Task 4.

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APPENDICES

1. Kotsopoulos J, Sohn K-J, Martin R, Renlund R, McKerlie C, Hwang S, Medline A, **Kim YI**. Dietary folate deficiency suppresses mammary tumorigenesis in a chemical carcinogen rat model of breast cancer. (poster presentation at Chemoprevention, P13-8 at Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orlando, Florida, September 25 – 28, 2002)
2. Kotsopoulos J, Sohn K-J, Martin R, Choi M, Renlund R, McKerlie C, Hwang S, Medline A, **Kim YI**. Dietary folate suppresses N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats. Carcinogenesis 2003; 24; 937 - 944

Appendix 1

DIETARY FOLATE DEFICIENCY SUPPRESSES MAMMARY TUMORIGENESIS IN A CHEMICAL CARCINOGEN RAT MODEL OF BREAST CANCER

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Epidemiological studies have suggested that dietary folate intake is inversely related to the risk of breast cancer. This study investigated the effect of dietary folate on the development and progression of N-methyl-N-nitrosourea (MNU)-induced mammary tumorigenesis in rats. Weanling, female Sprague-Dawley rats were randomized to receive an amino acid-defined diet containing either 0 mg (moderately folate deficient; n=22), 2 mg (basal dietary requirement [control]; n=20) or 8 mg (supplemented; n=20) folate/kg diet. At 50 days of age, all the rats received an intraperitoneal injection of MNU (50 mg/kg body weight) and the initial dietary interveniton was continued for additional 23 weeks. At necropsy, all macroscopic mammary tumors were identified and histologically confirmed for adenocarcinoma or its precursor, adenoma. Serum folate concentrations accurately reflected dietary folate levels at the time of MNU administration and at necropsy ($P<0.001$). The mean folate concentrations of the normal mammary gland of the folate-deficient group were significantly lower than those of the control and folate-supplemented groups ($P<0.001$), whereas no significant difference between the control and folate-supplemented groups was observed. The final incidence of mammary tumors in the folate-deficient group was significantly lower than that of the control and folate-supplemented groups (55% versus 90% and 75%, respectively, $P=0.04$). Kaplan-Meier analyses also demonstrated similar cumulative tumor incidence trends ($P=0.06$). By contrast, dietary folate supplementation did not significantly modulate both the final and cmulative incidences of mammary tumors compared with the control group. Dietary folate status had no significant effect on mean volume, weight, latency or multiplicity of mammary tumors. These data suggest that dietary folate deficiency of a moderate degree suppresses mammary tumorigenesis in this model. By contrast, dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis. The role of folate in mammaray tumorigenesis needs to be clarified in future studies for safe and effective prevention of breast cancer.

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Appendix 2

Dietary folate deficiency suppresses *N*-methyl-*N*-nitrosourea-induced mammary tumorigenesis in rats

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Epidemiologic studies have suggested that dietary folate intake is inversely related to breast cancer risk. However, epidemiologic evidence has not been consistent nor has it provided unequivocal support for this purported inverse relationship. This study investigated the effect of dietary folate on *N*-methyl-*N*-nitrosourea (MNU)-induced mammary tumorigenesis in rats. Weanling, female Sprague–Dawley rats were fed diets containing either 0 (deficient; $n = 22$), 2 (basal dietary requirement, control; $n = 20$) or 8 mg (supplemented; $n = 20$) folate/kg diet for 30 weeks. At 50 days of age, rats received an i.p. injection of MNU (50 mg/kg body wt). At necropsy, all macroscopic mammary tumors were identified and examined microscopically. The effect of dietary folate on genomic DNA methylation in mammary tumorigenesis was determined by the *in vitro* methyl acceptance assay. The incidence of mammary adenoma and adenocarcinoma in the folate-deficient group was lower than that of the control and folate-supplemented groups (55 versus 90 and 75%, respectively, $P = 0.043$). Kaplan–Meier analyses also demonstrated a similar trend in the rates of appearance of either adenoma or adenocarcinoma ($P = 0.06$). In contrast, folate supplementation did not significantly modulate mammary tumorigenesis compared with the control group. Although mammary tumors were significantly hypomethylated compared with non-neoplastic mammary tissues in each dietary group ($P < 0.03$), folate status did not significantly affect the extent of DNA methylation. The data suggest that dietary folate deficiency of a moderate degree suppresses, whereas folate supplementation at four times the basal dietary requirement does not significantly modulate, mammary tumorigenesis in this model. The role of folate in mammary tumorigenesis needs to be clarified for safe and effective prevention of breast cancer.

Introduction

Folate, a water-soluble B-vitamin and important co-factor in one-carbon metabolism, has recently been identified as an important nutritional factor that may modulate carcinogenesis (1–3). The role of folate in carcinogenesis has been best studied for colorectal cancer (1–3). The majority of over 25 published epidemiological studies indicate that dietary folate intake and blood folate levels are inversely associated with colorectal cancer risk (1–3). Although animal studies are generally supportive of a causal relationship between folate depletion and colorectal cancer risk, these studies have shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels (4–6) and folate intervention after microscopic neoplastic foci are established in the colorectal mucosa (7,8) promote, rather than suppress, colorectal carcinogenesis. An accumulating body of evidence suggests that folate status may also play a modulatory role in the development of several other malignancies (e.g. lung, pancreas, stomach, cervix, esophagus, brain and leukemia) (1–3). The precise nature and magnitude of the relationship between folate status and the risk of these malignancies, however, are less clearly defined compared with colorectal cancer.

The relationship between folate status and breast cancer risk has just begun to be reported in the epidemiological literature. Among nine published case-control studies that investigated the relationship between dietary folate intake and breast cancer risk, seven showed either a significant or equivocal inverse relationship that was not statistically significant, that became non-significant after adjustment, or that could not be distinguished from other factors in their relation to risk (9–15), whereas two showed an unequivocal null association (16,17). In some studies, the observed inverse association was further modified by the intake of alcohol and other folate co-factors (e.g. methionine, vitamins B_6 and B_{12}) (12,14,15). One nested case-control study, using stored serum samples, found no association between serum folate and breast cancer risk (18). Two large prospective studies have shown a weak inverse association between the total or dietary intake of folate and breast cancer risk (19,20). These prospective studies, however, have indicated that low intakes of folate increase, whereas high intakes of folate decrease, breast cancer risk among women who regularly consume alcohol (19,20), supporting folate–alcohol interactions in breast carcinogenesis observed in case-control studies (12,14,15). Recently, molecular epidemiologic studies have shown that the C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene may modulate breast cancer risk and that the direction and magnitude of the risk modification are influenced by folate status and alcohol consumption (21–23). MTHFR is a critical enzyme in folate metabolism that catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thereby playing an important role in DNA

Abbreviations: FPGS, folylpolyglutamate synthetase; MTHFR, methylenetetrahydrofolate reductase; MNU, *N*-methyl-*N*-nitrosourea.

synthesis, maintenance of nucleotide pool balance and DNA methylation (1). The MTHFR C677T polymorphism causes thermolability and reduced MTHFR activity, leading to lower levels of 5-methyltetrahydrofolate, an accumulation of 5,10-methylenetetrahydrofolate, increased plasma homocysteine levels (a sensitive inverse indicator of folate status), changes in cellular composition of one-carbon folate derivatives, and DNA hypomethylation (1).

Two animal studies published to date have suggested that folate may modulate mammary tumorigenesis. In mice with confirmed spontaneous mammary cancer, daily i.v. injections of fermentation *Lactobacillus casei* factor (pteroyltriglutamate) significantly regressed pre-existing mammary tumors and decreased new mammary tumor formation and lung metastases (24). Another study employing the *N*-methyl-*N*-nitrosourea (MNU) rat model showed that a folate-deficient diet provided during the initiation phase of mammary tumorigenesis significantly reduced tumor multiplicity and increased tumor latency compared with a control and folate-supplemented diet (25). The incidence of mammary tumors, however, was not significantly different among these groups (25). However, several inherent limitations associated with these animal studies including the use of non-standard dietary means to modulate folate status, possible growth retardation of animals, the concomitant use of antibiotics that may independently affect folate levels, and the use of animals that are resistant to chemically induced mammary tumorigenesis preclude a definitive conclusion concerning the effect of folate on mammary tumorigenesis.

Because only few modifiable risk factors for breast cancer exist, recent epidemiological observations which suggest that folate deficiency increases, whereas supplementation reduces, breast cancer risk merit further consideration. Folate is an ideal agent for potential chemoprevention of breast cancer. It is a natural vitamin, inexpensive, virtually free of side effects (26) and possesses biologically plausible mechanisms for cancer prevention (1–3). However, the results from published epidemiological and animal studies have been neither consistent nor convincing. Furthermore, a growing body of evidence suggests that folate possesses the dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention (4–8,27,28). Folate deficiency has an inhibitory, whereas folate supplementation has a promoting, effect on progression of established neoplasms (4–8,27,28). In contrast, folate deficiency in normal epithelial tissues appears to predispose them to neoplastic transformation, and modest levels of folate supplementation suppress the development of tumors in normal tissues (4–8,27,28). Therefore, the potential effect of folate chemoprevention needs to be clearly elucidated in appropriate animal models before folate supplementation can be considered in humans. Given these considerations, this study investigated the effects of dietary folate deficiency and supplementation on the development and progression of mammary tumors in the well-established MNU rat model of breast cancer. Given the role of folate in DNA methylation, an important epigenetic determinant in carcinogenesis (29,30), we also investigated whether dietary folate modulates genomic DNA methylation in MNU-induced mammary tumorigenesis. Folate, in the form of 5-methyltetrahydrofolate, is involved in remethylation of homocysteine to methionine, which is a precursor of *S*-adenosylmethionine (SAM), the primary methyl group donor for most biological methylation reactions (1–3).

Materials and methods

Animals and dietary intervention

This study was approved by the Animal Care Committee of the University of Toronto. Pathogen-free, weanling female Sprague–Dawley rats (~50 g; Charles River Laboratories, St Constant, Quebec, Canada) were randomly assigned to receive an amino acid-defined diet (Dyets, Bethlehem, PA) (31) containing either 0 ($n = 22$), 2 ($n = 20$) or 8 ($n = 20$) mg folic acid/kg diet from weaning at 3 weeks of age for 27 weeks through the MNU treatment (at 50 days of age). Rats were singly housed and maintained at 24 ± 2°C at 50% humidity with a 12 h light/dark cycle. These diets constitute a standard method of inducing folate deficiency or providing supplemental dietary folate in rodents (31) and have been utilized extensively in previous studies of folate and colorectal cancer (4,7,8,27). The diet containing 0 mg folic acid/kg produces progressive folate deficiency of a moderate degree without anemia, growth retardation or premature death through weeks 3–5, after which systemic folate indicators stabilize (27). Although this diet is completely devoid of folate, severe folate deficiency is not induced because of de novo synthesis of folate by intestinal bacteria, some of which is incorporated into the tissue folate of the host (32). This folate-deficient diet is identical to that associated with an increased risk of colorectal neoplasms in previous animal studies using a chemical colorectal carcinogen or genetically engineered murine models of colorectal cancer (4,7,8,27). Two milligram folic acid per kilogram diet is generally accepted as the basal dietary requirement for rodents (33). The diet containing 8 mg folic acid/kg represents folate supplementation four times the basal dietary requirement. This level of folate was chosen because the 8 mg/kg level has consistently provided a degree of chemoprevention against colorectal cancer in previous rodent studies (4,7,27). These diets contained 50 g cellulose/kg, 60% of the calories as carbohydrates, 23% as fat (or 10% by weight), and 17% as L-amino acids (31). The amount of methyl donors, methionine, choline and vitamin B_{12}, 8.2 g, 2.0 g and 50 μg/kg diet, respectively. The detailed composition of the diets has been published previously (8,31). Diets and water were provided *ad libitum*.

MNU administration

Notwithstanding the limitations associated with animal models, the MNU rat model is widely used to determine the effects of dietary factors on mammary tumorigenesis for the following reasons: (i) histological similarities of adenocarcinoma to human breast cancer; (ii) local invasiveness and metastatic potential; (iii) a clear operational distinction between the initiation and promotion stages; and (iv) hormonally dependent mammary tumorigenesis (34–38). At 50 days of age, all rats received one i.p. injection of MNU (50 mg/kg body wt; Sigma Chemical, St Louis, MO). A single injection of 50 mg MNU/kg has become the standard dosage due to its rapid induction and high incidence of mammary tumors combined with minimal toxicity and a short latency period of 3–6 months (34,35).

Observation parameters

Body weights were recorded weekly. The daily food consumption of each group was measured on a predetermined day of each week. All rats were palpated for mammary tumors once a week beginning 4 weeks after MNU administration. The number, size and location of each tumor were recorded in a manner that, after histological diagnosis, the time of appearance of the cancers could be determined. All the rats were monitored daily for clinical evidence of illness or morbidity and those approaching a moribund state were promptly killed. In addition, rats with tumor burden exceeding 10% of body weight, tumors >15–20 mm in diameter, tumors that impaired normal movement of the animals and ulcerating tumors were immediately killed during the study.

Sample collection and analysis of mammary tumors

Blood was withdrawn from the lateral tail vein of each rat within a week of MNU injection and from the heart at necropsy and centrifuged at 5000 r.p.m. for 10 min at 4°C. Serum was stored at −70°C in 0.5% ascorbic acid for serum folate assay. Given the latency period of 3–6 months associated with a single i.p. MNU injection and the average duration for the systemic and tissue folate levels to stabilize, the rats were killed by carbon dioxide inhalation followed by cervical dislocation at 23 weeks after MNU injection (27 weeks after dietary intervention or 30 weeks of age). The liver from each rat was harvested, snap-frozen and stored at −70°C for determination of hepatic folate concentration. All macroscopic mammary tumors were counted, excised and weighed, and the diameter of each tumor was measured using a digital caliper for final tumor volume computation in a blinded fashion. One-half of each macroscopic tumor was snap-frozen in liquid nitrogen and stored at −70°C for DNA extraction. The other half of the tumor was fixed in 10% neutral-buffered formalin, processed in a standard manner for hematoxylin–eosin (H&E) staining and histologically analyzed according to Russo *et al.* (37) by three experienced pathologists (R.R., C.M. and A.M.) blinded to the study group independently.

In the case of a discrepancy, two similar interpretations were utilized for the final analysis. Normal mammary tissue was also excised at necropsy from each rat, snap-frozen in liquid nitrogen and stored at −70°C for DNA extraction and mammary tissue folate determination.

Determination of folate concentration

Serum folate concentrations were determined by a standard microbiological microtiter plate assay using *L.casei* (39). Hepatic and normal mammary tissue folate concentrations were measured by the same microbiologic assay (39), utilizing a previously described method for the determination of tissue folates (40).

DNA extraction

DNA from normal mammary tissue and mammary tumors was extracted by standard technique using a lysis buffer containing proteinase K followed by phenol, chloroform and isoamyl alcohol organic extraction (41). The size of DNA estimated by agarose-gel electrophoresis was >20 kb in all instances. No RNA contamination was detected on agarose-gel electrophoresis. The final preparations had a ratio of A_{260} to A_{280} between 1.8 and 2.0. The concentration of each DNA sample was determined as the mean of three independent spectrophotometric readings.

Genomic DNA methylation determination

The methylation status of cytosine-guanine (CpG) sites in genomic DNA from normal mammary tissue and mammary tumors was determined by the *in vitro* methyl acceptance capacity of DNA using ^{3}H-methyl-SAM as a methyl donor and a prokaryotic CpG DNA methyltransferase, Sss1, as described previously (4,8,42,43). The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous ^{3}H-methyl incorporation. Briefly, mammary tumor and non-neoplastic mammary gland DNA (500 ng) was incubated with 2.0 μCi of ^{3}H-methyl-SAM (New England Nuclear, Boston, MA), 3 U Sss1 methylase (New England Biolabs, Beverly, MA), and 1× Sss1 methylation buffer [120 mM NaCl, 10 mM Tris–HCl (pH 7.9), 10 mM EDTA, 1 mM dithiothreitol] in a total volume of 30 μl for 1 h at 30°C. The Sss1 was inactivated by incubating at 65°C for 10 min. The *in vitro* methylated DNA was isolated from a 15 μl aliquot of the reaction mixture by filtration on a Whatman DE-81 ion-exchange filter (Fisher Scientific, Springfield, NJ). The DNA was washed three times with 0.5 M sodium phosphate buffer (pH 7.0), air-dried and the radioactivity of the DNA retained in the filters was measured by scintillation counting using a non-aqueous scintillation fluor. The amount of radiolabel bound to a filter from an incubation mixture without DNA (control) was used as background and was subtracted from the values obtained with mixtures containing DNA. The background value was always <1% of the uptake observed with DNA samples. All analyses were performed in duplicate.

Statistical analysis

Between-group comparisons of continuous variables were assessed using the Kruskal–Wallis and Mann–Whitney non-parameteric tests. For categorical response variables, differences among the groups were assessed by Pearson χ^2. Differences in genomic DNA methylation between normal mammary gland and tumor in each diet group was assessed by the Wilcoxon signed ranks test. The Kaplan–Meier survival analysis and the Log Rank test were used to compare the rates of tumor appearance among the three groups. All significance tests were two sided and were considered statistically significant if the observed significance level was <0.05. Results are expressed as mean ± SEM. Statistical analyses were performed using SPSS (version 10).

Results

Body weight and daily food consumption

Growth curves were similar among the three dietary groups; at no time point did the mean body weights differ significantly among the three groups. This finding indicates that folate deficiency in the rats fed 0 mg folate/kg diet was moderate; otherwise, growth retardation or premature death would have occurred (44). The mean daily food consumption, which was determined on a pre-assigned day of each week, was also similar among the three groups.

Serum, liver and normal mammary gland folate concentrations

At the time of MNU injection (4 weeks after the start of dietary intervention) and at necropsy (27 weeks after the start of dietary intervention), the mean serum folate concentrations were significantly different among the three groups ($P < 0.001$; Table I). The mean serum folate concentrations of the three dietary groups at these two time points were comparable with those observed in rats and mice placed on the corresponding diets for 20–24 weeks in previous studies (4,7,27,45). These observations indicate that a sufficient degree of systemic folate deficiency and supplementation was achieved in the folate-deficient and supplemented rats, respectively, at the time of MNU injection and throughout the study period for the determination of the effect of folate status on MNU-induced mammary tumorigenesis. At necropsy, the hepatic folate concentrations of the three dietary groups were significantly different ($P < 0.001$; Table I), and these levels were comparable with those observed in rats placed on the corresponding diets for 24 weeks in previous studies (27,46). At necropsy, the mean mammary gland folate concentration of the folate-deficient group was significantly lower than the control and folate-supplemented groups ($P < 0.001$) while no significant difference was observed between the control and folate-supplemented groups (Table I). This observation suggests that mammary gland folate concentrations reached a plateau beyond the 2 mg folate/kg diet. This finding is probably due to the fact that folate accumulation in tissues is limited by the level of folylpolyglutamate synthetase (FPGS) activity in the setting of substrate excess (47,48).

Effects of dietary folate on MNU-induced mammary tumorigenesis

No rats died prematurely or were killed before necropsy in the three dietary groups for reasons other than the presence of large and/or ulcerating tumors as defined in the Materials and methods section. The prevalence of killed rats was similar among the three groups. Consistent with previous observations made in the MNU-Sprague–Dawley rat model of mammary tumorigenesis (34–38), >90% of macroscopic mammary tumors in the present study were identified histologically as either adenomas (15%) or adenocarcinomas (85%). There was an excellent agreement in histological diagnosis of either adenoma or adenocarcimona among the three study pathologists (kappa statistic = 0.95). The analyses pertaining to mammary tumors were performed for the combination of

Table I. Serum, hepatic and mammary gland folate concentrations*

	At the time of MNU injection (4 weeks of dietary intervention)			At necropsy (27 weeks of dietary intervention)		
Diet (mg folate/kg diet)	0	2	8	0	2	8
Serum folate (ng/ml)	13.75 ± 1.04[a]	68.82 ± 2.65[b]	100.78 ± 2.82[c]	10.53 ± 1.16[a]	49.24 ± 3.16[b]	77.35 ± 2.33[c]
Hepatic folate (μg/g tissue)				2.18 ± 0.32[a]	6.42 ± 0.35[b]	9.01 ± 0.37[c]
Mammary folate (ng/g tissue)				56.20 ± 7.54[a]	178.13 ± 29.06[b]	175.94 ± 24.32[b]

*Results are expressed as mean ± SEM. Means in a row with different letters at each time point significantly differ at $P < 0.001$.

adenocarcinomas and adenomas and for adenocarcinomas alone. There were not a sufficient number of adenomas for independent analysis.

As shown in Figure 1A, there was a trend towards a significant difference in the rates of appearance of either adenocarcinomas or adenomas among the three dietary groups ($P = 0.07$). This was mainly due to the difference between the folate-deficient and control groups ($P = 0.02$). In contrast, there was no significant difference between the folate-deficient and supplemented groups ($P = 0.11$), and between the control and folate-supplemented groups ($P = 0.72$). We excluded one outlier from the folate-deficient group, which harbored a total of nine adenocarcinomas and adenomas, and this strengthened the overall comparison ($P = 0.06$). When the analysis was confined to adenocarcinomas alone, similar patterns were observed. There was a trend towards a significant difference in the rates of appearance of adenocarcinomas among the three groups (P-overall $= 0.08$; $P = 0.05$ between the folate-deficient and control groups; $P = 0.04$ between the folate-deficient and supplemented groups; $P = 0.83$ between the control and folate-supplemented groups; Figure 1B).

There was a trend towards a significant difference in the final incidence of histologically confirmed adenocarcinomas and adenomas at necropsy ($P = 0.057$; Table II). This was mainly due to the difference between the folate-deficient and control groups ($P = 0.02$); there was no significant difference between the folate-deficient and supplemented groups ($P = 0.19$) or between the control and folate-supplemented groups ($P = 0.20$). When the outlier was excluded from the folate-deficient group, the overall difference in the incidence of adenocarcinomas and adenomas became significant ($P = 0.043$) with a similar trend in between-group comparisons. As shown in Table II, there was no significant difference in mean tumor latency (mean time to appearance of first palpable tumor), multiplicity (mean number of tumors per tumor-bearing rat), volume or weight among the three groups, whether or not the outlier was excluded in the analyses. When the analyses were confined to adenocarcinomas alone, no

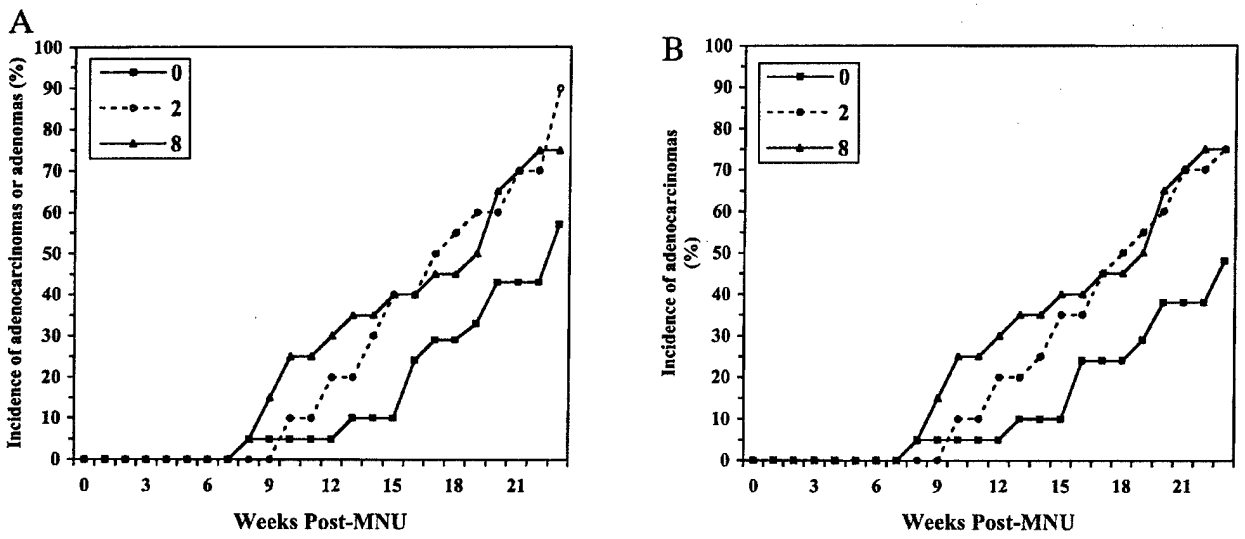

Fig. 1. (A) The rate of appearance of either mammary adenomas or adenocarcinomas among the three dietary groups (P-overall $= 0.07$; $P = 0.02$ between the 0 and 2 mg folic acid groups; $P = 0.11$ between the 0 and 8 mg folic acid groups; $P = 0.72$ between the 2 and 8 mg folic acid groups by the Kaplan–Meier survival analysis and Log Rank test). Excluding one outlier in the 0 mg folic acid group, which harbored a total of nine adenocarcinomas and adenomas, strengthened the overall comparison ($P = 0.06$) with similar patterns in between-groups comparisons ($P = 0.02$ between the 0 and 2 mg folic acid groups; $P = 0.09$ between the 0 and 8 mg folic acid groups; $P = 0.72$ between the 2 and 8 mg folic acid groups). (B) The rate of appearance of mammary adenocarcinomas among the three dietary groups (P-overall $= 0.08$; $P = 0.05$ between the 0 and 2 mg folic acid groups; $P = 0.04$ between the 0 and 8 mg folic acid groups; $P = 0.83$ between the 2 and 8 mg folic acid groups by the Kaplan–Meier survival analysis and Log Rank test).

Table II. Effects of dietary folate on the incidence, latency, multiplicity, volume and weight of mammary adenocarcinomas and adenomas*

Diet (mg folate/kg diet)	0	2	8	P-value, ANOVA
Incidence (%)	57^{a}	90^{b}	$75^{a,b}$	0.057
Incidence (%)†	55^{a}	90^{b}	$75^{a,b}$	0.043
Mean latency (weeks post-MNU injection)	17.83 ± 1.35	17.06 ± 1.11	15.00 ± 1.36	0.29
Mean multiplicity	3.67 ± 1.03	2.87 ± 0.53	2.20 ± 0.34	0.72
Mean volume (cm^3)	2.83 ± 0.80	2.88 ± 0.58	1.49 ± 0.39	0.28
Mean weight (g)	0.86 ± 0.23	1.03 ± 0.23	0.58 ± 0.14	0.45

*Results are expressed as mean ± SEM. Means in a row with different letters significantly differ at $P < 0.02$ by between-group comparisons.
†Excluding one outlier in the 0 mg folate group, which harbored a total of nine adenocarcinomas and adenomas, strengthened the overall comparison of the incidence of adenocarcinomas and adenomas among the three groups. In contrast, no significant difference in mean latency, multiplicity, volume and weight of adenocarcinomas and adenomas was observed among the three groups whether or not the outlier was included or excluded in the analyses.

Table III. Effects of dietary folate on the incidence, latency, multiplicity, volume and weight of mammary adenocarcinomas*

Diet (mg folate/kg diet)	0	2	8	*P*-value, ANOVA
Incidence (%)	48	75	75	0.10
Mean latency (weeks post-MNU injection)	17.40 ± 1.54	16.27 ± 1.10	15.00 ± 1.36	0.51
Mean multiplicity	3.71 ± 1.04	3.00 ± 0.59	1.90 ± 0.29	0.21
Mean volme (cm^3)	3.15 ± 0.99	3.10 ± 0.65	1.64 ± 0.42	0.38
Mean weight (g)	0.98 ± 0.28	1.12 ± 0.25	0.63 ± 0.15	0.42

*Results are expressed as mean ± SEM. No significant difference in mean latency, multiplicity, volume and weight of adenocarcinomas was observed among the three groups.

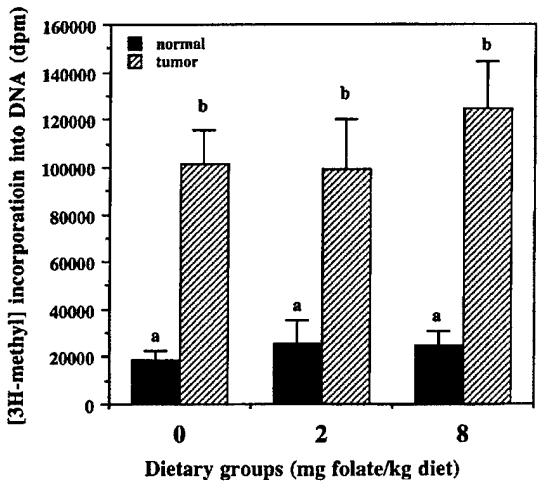

Fig. 2. Effects of dietary folate on genomic DNA methylation in mammary adenocarcinomas and non-neoplastic mammary tissues as determined by the *in vitro* methyl acceptance assay. The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous ^{3}H-methyl incorporation into DNA. Different letters within each dietary group denote significant differences by the Wilcoxon signed ranks test at $P < 0.03$. Values are mean ± SEM.

significant difference in the final incidence and mean tumor latency, multiplicity, volume or weight among the three groups was observed (Table III).

Genomic DNA methylation status

As shown in Figure 2, the degree of ^{3}H-methyl incorporation into DNA of the mammary adenocarcinoma and into DNA from the pair-matched non-neoplastic mammary tissue was not significantly different among the three dietary groups. However, the degree of ^{3}H-methyl incorporation into DNA of the mammary adenocarcinomas, which is inversely related to the extent of genomic DNA methylation, was 4–5-fold higher than that of non-neoplastic mammary tissue within each dietary group ($P < 0.03$; Figure 2), indicating a significantly lower degree of genomic DNA methylation in adenocarcinomas compared with normal mammary tissue.

Discussion

Our data suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats. In contrast, dietary folate supplementation at four times the basal dietary requirement does not appear to modulate mammary tumorigenesis in this model. These observations contradict the generally accepted notion based on epidemiologic evidence, which suggests that folate deficiency enhances, whereas folate supplementation suppresses, the development of breast cancer (9–15,19,20). Epidemiologic evidence available thus far has not been consistent nor has it provided unequivocal support for the purported inverse relationship between folate status and breast cancer risk (9–20). However, none of the published epidemiologic studies has demonstrated a positive association between folate status and breast cancer risk. Some epidemiological studies have suggested that folate status alone may not be sufficient in modifying breast cancer risk. However, with alcohol consumption folate deficiency potentiates, whereas folate supplementation reduces, the risk of breast cancer (12,14,15,19,20). Furthermore, some sudies have suggested that folate status may modify breast cancer risk in conjunction with other dietary factors involved in one-carbon metabolism such as methionine, vitamins B_6 and B_{12} (15,19). Also, there is evidence that the direction and magnitude of the breast cancer risk modification associated with folate status may depend on the MTHFR C677T polymorphism (21–23).

Our data differ from the promoting and protective effect of folate deficiency and supplementation, respectively, on intestinal tumorigenesis observed in the chemical carcinogen (dimethylhydrazine) and genetically engineered rodent models utilizing the same diets employed in the present study (4,7,27,45). However, some animal studies have suggested that folate status may have the opposite effect on intestinal tumorigenesis depending on the timing and dose of folate intervention (4–8). The contradicting effect of dietary folate on mammary and intestinal tumorigenesis in animal models using the same diets suggests that folate may modulate carcinogenesis in a tissue- and/or carcinogen-specific manner. The results from the present study are, however, consistent with those of a previous study that investigated the effect of dietary folate deficiency and supplementation on initiation and early promotion of MNU-induced mammary tumorigenesis in Fischer 344 rats (25). Baggott and colleagues performed a study in which rats were fed a casein-based AIN-76A diet containing either 0, 2 or 40 mg folic acid/kg diet, or 20 mg folinic acid/kg diet at weaning (27 days of age) for 30 days, injected with MNU intravenously (50 mg/kg body wt), and subsequently fed the control diet containing 2 mg folic acid/kg for 180 days. Glycine and succinylsulfathiazole (10 g/kg diet) were added to the diet to potentiate folate deficiency. Plasma folate concentrations were 15 ± 5, 77 ± 15 and 218 ± 47 ng/ml for the 0, 2 and 40 mg folic acid/kg diet groups at the time of MNU injection and 79 ± 8, 58 ± 6 and 56 ± 6 ng/ml at necropsy. Although the incidence of mammary cancer was not significantly different among the four groups,

cancer multiplicity was significantly lower in rats fed the 0 mg folic acid diet than those fed the 2 mg folic acid, the 40 mg folic acid or the 20 mg folinic acid diets; there was no significant difference in cancer multiplicity among the latter three groups. Furthermore, the time required for 50% of the rats to develop palpable mammary tumors was significantly longer in the 0 mg folic acid group than in the 40 mg folic acid or the 20 mg folinic acid group, but was not significantly different from that in the 2 mg folic acid group. Thus, Baggott's study demonstrated that folate deficiency suppressed initiation and early promotion of MNU-induced mammary tumorigenesis (25).

As suggested by Baggott's study (25), the inhibitory effect of folate deficiency on MNU-induced mammary tumorigenesis in rats may be a real effect on initiation and early promotion. However, it is possible that the conventional dose and route of MNU injection employed in the present study might have created an overwhelmingly carcinogenic milieu for folate status to modulate initiation of mammary tumorigenesis. Regardless of the levels of dietary folate, MNU probably induced and established neoplastic foci in mammary tissues. In this setting, folate deficiency probably suppressed the progression of and/or caused regression of established mammary neoplastic foci. This explanation is consistent with the biochemical function of folate. Interruption of folate metabolism in rapidly replicating neoplastic cells to cause ineffective DNA synthesis and hence the inhibition of tumor growth has been the basis of antitumor therapy using antifolate agents (49). Folate deficiency has been shown to induce regression and suppress progression of pre-existing neoplasms in experimental models (4,7,8,50–52). Therefore, it is possible that the inhibitory effect of folate deficiency on MNU-induced tumorigenesis in this rat model might have been primarily on promotion/progression of established mammary neoplastic foci. In this regard, although Baggott's study was primarily designed to test the effect of folate on initiation and early promotion, it is possible that the observed effect of folate was actually on promotion/progression because of the dose and route of MNU employed in that study (25).

In the present study, dietary folate supplementation at four times the basal dietary requirement, which has consistently conferred protection against intestinal tumorigenesis in rodents in previous studies (4,7,8,27), did not inhibit mammary tumorigenesis. This level of dietary folate supplementation did not promote the progression of MNU-induced mammary neoplastic foci in the present study in contrast to the promoting effect associated with this level of dietary folate supplementation on progression of established intestinal neoplastic foci observed in some studies (7,8). The lack of effect associated with folate supplementation on mammary tumorigenesis in the present study may be related to the fact that, in spite of significantly higher serum and hepatic folate levels, the mean mammary gland folate concentration of the folate-supplemented rats was not significantly different from that of the controls. Previous studies have demonstrated a dose–responsive tissue saturating effect of folate supplementation above four times the basal dietary requirement in rat colon (4), and the 8 mg folic acid diet has consistently induced significantly higher colonic mucosal folate concentrations compared with the 2 mg folic acid (control) diet in rodents (4,7,8,46,53). It is well known that different tissues express different folate requirements and hence different susceptibility to folate deficiency (40). Furthermore, folate accumulation in tissues is limited by the level of FPGS activity in the setting of substrate excess (47,48). FPGS catalyzes polyglutamation of intracellular folates, thereby allowing the retention of folate that would otherwise be lost because of efflux from the cell (47,48). Previous studies in animals and in cultured cells have shown that tissue levels of folate reach a plateau when FPGS is saturated from excess folate in the diet or culture medium (4,47,48). At present, there is no information in the literature regarding the levels of FPGS activity in normal mammary tissue. It is possible that the levels of FPGS activity in mammary gland are appreciably lower than the liver or colon and thus tissue folate is saturated at a much lower level of dietary folate in mammary gland compared with other tissues. However, it is also possible that higher levels of dietary folate supplementation above four times the basal dietary requirement may be necessary to increase mammary folate concentrations compared with the control diet.

One interesting finding in this study is that the extent of genomic DNA methylation is significantly lower in mammary adenocarcinomas than in non-neoplastic mammary tissues regardless of folate status. DNA methylation is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in chromatin modifications and in the development of mutations (29,30). Neoplastic cells simultaneously harbor widespread genomic DNA hypomethylation and more specific regional areas of hypermethylation (29,30). Genomic hypomethylation is an early, and consistent, event in carcinogenesis and is associated with genomic instability and increased mutations (29,30). Site-specific hypomethylation at the promoter region of tumor suppressor and mismatch repair genes is an important mechanism in gene silencing in carcinogenesis (29,30). Although promoter CpG islands hypermethylation and consequent inactivation of several tumor suppressor genes have been observed in human breast cancer (54), very few studies have reported genomic hypomethylation in human breast cancer (55,56). To our knowledge, our study is the first to demonstrate that genomic DNA hypomethylation is an epigenetic phenomenon associated with MNU-induced mammary tumorigenesis in rats. The extent of genomic DNA methylation in mammary adenocarcinomas and in non-neoplastic mammary tissues was not significantly modulated by folate status. This observation suggests that altered genomic DNA methylation was not a probable mechanism by which folate deficiency suppressed mammary tumorigenesis in our study. Because folate may modulate DNA methylation in a site-specific manner (43), however, the possibility that folate status may affect site-specific methylation of critical genes implicated in mammary tumorigenesis cannot be ruled out in the present study.

The strengths of the present study include: (i) the use of the amino acid-defined diet that is widely accepted as the standard means of inducing folate deficiency or providing supplemental dietary folate in rodents; (ii) the use of dietary levels of folate that have been shown to modulate development of other cancers in this strain of rats; (iii) measurements of systemic and mammary gland folate concentrations; (iv) rigorous histological confirmation of all mammary tumors to ensure an accurate determination of the rate of appearance and other tumor-specific parameters of adenomas and adenocarcinomas. However, several limitations associated with the present study need to be acknowledged. First, although the dose and route of MNU administration employed in the present study may be appropriate in studies examining the effect of other potential

chemopreventive agents in this model, the effect may be too overwhelmingly carcinogenic for folate to modulate. Therefore, the effect observed with dietary folate in the present study may be predominantly on promotion and progression, and not on initiation, of MNU-induced neoplastic foci. Secondly, the fat content of the diets used in the present study was higher than the AIN rodent diets that are more commonly used in experimental mammary tumor studies (10 versus 7% by weight). Animal studies have generally suggested that high fat diets enhance mammary tumorigenesis in rodents (57). Therefore, it is possible that the tumor-promoting effect associated with the higher fat content in our diets might have masked any modulating effect of dietary folate intervention. Thirdly, the mean mammary gland folate concentration associated with folate supplementation was not significantly higher than that of the control diet. Therefore, higher levels of folate supplementation above four times the basal dietary requirement may be necessary to significantly increase mammary gland folate concentrations and to observe any modulatory effect of folate supplementation on mammary tumorigenesis. Lastly, the number of animals employed in the present study did not allow us to achieve adequate statistical power. It would have required 103 animals in total to be 80% certain of detecting a 35% reduction in tumor incidence associated with folate deficiency compared with the control diet at a 5% level of significance.

In summary, our data suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats. In contrast, dietary folate supplementation at four times the basal dietary requirement does not significantly modulate mammary tumorigenesis. Notwithstanding the limitations associated with this model, our data suggest that the role of folate in mammary tumorigenesis needs to be clarified in future studies for safe and effective prevention of breast cancer.

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